IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	: Confirmation No. 7978
Serial No. 10/559,835	: Group Art Unit 1633
Takehisa Matsuda	: Attorney Docket No. 2005_1807A
Filed : March 8, 2006	: Examiner LEAVITT, MARIA GOMEZ

DECLARATION UNDER 37 CFR 1.132

(No.2)

Honorable Commissioner of Patents and Trademarks

Sir:

I, Kunio MATSUMOTO hereby declare that:

I was born in Nagano prefecture, Japan, in 1959;

I am a citizen of Japan and a resident of 2-9-3-303, Hiro-oka, Kanazawa City, Kanazawa 920-0031 JAPAN;

I graduated from Department of Biology, Faculty of Science, Kanazawa University, Japan in 1981.

I received my doctor degree on the study of "Analysis of intermolecular relationship in photosynthetic oxygen evolving complex" at Osaka University, Japan, in 1986;

I have worked as an Associate Professor of Osaka University in Japan from 1990-2007 and as a Professor of Kanazawa

University from 2007 until now and have engaged in a study on hepatocyte growth factor and NK4;

I am one of the inventors for this application;

I have many reports relating to HGF and NK4. A part of my reports are as follows:

1: Matsumoto, K., and Nakamura, T.: Hepatocyte growth factor (HGF) as tissue organizer for organogenesis and regeneration. *Biochem. Biophys. Res. Commun.*, 239, 639-644, 1997.

2: Matsumoto, K., and Nakamura, T.: Mechanisms and significance of bifunctional NK4 in Cancer Treatment. *Biochem. Biophys. Res. Commun.*, 333: 316-327, 2005.

3: Matsumoto, K., Nakamura, T., Sakai, K., and Nakamura, T.: Hepatocyte Growth Factor and Met in Tumor Biology and Therapeutic Approach with NK4. *Proteomics*, 8: 3360-3370, 2008.

4: Date, K., Matsumoto, K., Shimura, Tanaka, H. H. M. and Nakamura, T.: HGF/NK4 is a specific antagonist for pleiotrophic actions of hepatocyte growth factor. *FEBS Lett.*, 420, 1-6, 1997.

5: Date, K., Matsumoto, K., Kuba, K., Shimura, H., Tanaka, M. and Nakamura, T.: Inhibition of tumor growth and invasion by a four-kringle antagonist (HGF/NK4) for hepatocyte growth factor. *Oncogene*,17: 3045-3054, 1998.

6: Matsumoto, K., Kataoka, H., Date, K. and Nakamura, T.: Cooperative interaction between α-chain and β-chain of HGF on c-Met receptor confers ligand-induced receptor tyrosine phosphorylation and multiple biological responses. *J. Biol. Chem.*, 273, 22913-22920, 1998

7: Kuba, K., Matsumoto, K., Date, K., Shimura, H., Tanaka, M., and Nakamura, T.: HGF/NK4, a four-kringle antagonist of hepatocyte growth factor, is an angiogenesis inhibitor that suppress tumor growth and metastasis in mice. *Cancer Res.*, 60: 6737-6743, 2000.

8: Tomioka, D., Maehara, N., Kuba, K., Mizumoto, K., Tanaka, M., Matsumoto, K., and Nakamura, T.: Inhibition of growth, invasion, and metastasis of human pancreatic carcinoma cells

by NK4 in an orthotopic mouse model. *Cancer Res.*, 61: 7518-7524, 2001.

9: Maemondo, M., Narumi, K., Saijo, Y., Usui, K., Yahara, M., Tazawa, R., Hagiwara, K., Matsumoto, K., Nakamura, T., and Nukiwa, T.: Targeting angiogenesis and HGF function using an adenovirul vector expressing the HGF-antagonist NK4 for cancer therapy. *Mol. Therapy*, 5: 177-185, 2002.

10: Kikuchi, T., Maemondo, M., Narumi, K., Matsumoto, K., Nakamura, T., and Nukiwa, T.: Tumor suppression induced by intratumor administration of adenovirus vector expressing NK4, a 4-kringle antagonist of hepatocyte growth factor, and naïve dendritic cells. *Blood*, 100: 3950-3959, 2002.

11: Martin, T. A., Parr, C., Davies, G., Watkins, G., Lane, J., Matsumoto, K., Nakamura, T., Mansel, R. E., and Jiang, W. G.: Growth and angiogenesis of human breast cancer in a nude mouse tumour model is reduced by NK4, the HGF/SF antagonist. *Carcinogenesis*, 24: 1317-1323, 2003.

12: Manabe, T., Mizumoto, K., Nagai, E., Matsumoto, K., Nakamura, T., Nukiwa, T., Tanaka, M., and Matsuda, T.: Cell-based protein delivery system for the inhibition of the growth of pancreatic cancer: NK4 gene-transduced oral mucosal epithelial cell sheet. *Clin. Cancer Res.*, 9: 3158-3166, 2003.

13: Davies, G., Mason, M. D., Martin, T. A., Parr, C., Watkins, G., Lane, J., Matsumoto, K., Nakamura, T., and Jiang, W. G.: The HGF/SF antagonist NK4, reverses fibroblast- and HGF-induced prostate tumor growth and angiogenesis in vivo. *Int. J. Cancer*, 106: 348-354, 2003.

14: Wen, J., Matsumoto, K., Taniura, N., Tomioka, D., and Nakamura, T.: Hepatic gene expression of NK4, an HGF-antagonist/angiogenesis inhibitor, suppresses liver metastasis and invasive growth of colon cancer in mice. *Cancer Gene Therapy*, 11: 419-430, 2004.

15: Murakami, M., Nagai, E., Mizumoto, K., Saimura, M., Ohuchida, K., Inadome, N., Matsumoto, K., Nakamura, T., Maemondo, M., Nukiwa, T., and Tanaka, M.: Suppression of metastasis of human pancreatic cancer to the liver by transportal injection of recombinant adenoviral NK4 in nude mice. *Int. J. Cancer*, 117: 160-165, 2005.

16: Namiki, Y., Namiki, T., Yoshida, H., Date, M., Yashiro, M., Matsumoto, K., Nakamura, T., Yanagihara, K., Tada, N., Satoi, J., and Fujise, K.: Preclinical study of a "tailor-made" combination of NK4-expressing gene therapy and gefitinib (ZD1839, Iressatrade mark) for disseminated peritoneal scirrhous gastric cancer. *Int. J. Cancer*, 118:1545-1555, 2006.

17: Ogura, Y., Mizumoto, K., Nagai, E., Murakami, M., Inadome, N., Saimura, M., Matsumoto, K., Nakamura, T., Maemondo, M., Nukiwa, T., and Tanaka, M.: Peritumoral injection of adenovirus vector expressing NK4 combined with gemcitabine treatment suppresses growth and metastasis of human pancreatic cancer cells implanted orthotopically in nude mice and prolongs survival. *Cancer Gene Ther.*, 13: 520-529, 2006.

18: Son, G., Hirano, T., Seki, E., Iimuro, Y., Nukiwa, T., Matsumoto, K., Nakamura, T., and Fujimoto, J.: Blockage of HGF/c-Met system by gene therapy (adenovirus-mediated NK4 gene) suppresses hepatocellular carcinoma in mice. *J. Hepatol.*, 45: 688-695, 2006.

19: Du, W., Hattori, Y., Yamada, T., Matsumoto, K., Nakamura, T., Sagawa, M., Otsuki, T., Niikura, T., Nukiwa, T., and Ikeda, Y.: NK4, an antagonist of hepatocyte growth factor (HGF), inhibits growth of multiple myeloma cells in vivo; molecular targeting of angiogenic growth factor. *Blood*, 109: 3042-3049, 2007.

20: Kishi, Y., Kuba, K., Nakamura, T., Wen, J., Suzuki, Y., Mizuno, S, Nukiwa, T., Matsumoto, K, and Nakamura, T. Systemic NK4 gene therapy inhibits tumor growth and metastasis of melanoma and lung carcinoma in syngeneic mouse tumor models. *Cancer Sci.*, 100: 1351-1358, 2009.

The experiments given below were conducted under my supervision.

Experiment

(A) Method

(1) Production of NK4 by epithelial cells of oral mucosa

The experiment is Test Example 1 of the specification of the present application.

(1-1) Preparation of NK4 cDNA

mRNA was isolated from subcutaneous tissue cells of Wister rat or OMEC using ISOGEN-LS (Nippon Gene Co., Ltd., Toyama, Japan), and the mRNA was used for RT-PCR(reverse transcription/polymerase chain reaction) to isolate NK4 cDNA. Specifically, 0.5 μl of mRNA solution (150 ng of mRNA), and 5 μl of 10×RT-PCR solution (500 mM KCl, 100 mM Tris-HCl (pH 9.0), 1% Triton X-100, 15 mM $MgCl_2$), 4 μl of dNTP (2.5 mM), 2 μl of primer 1 (10 mM), 2 μl of primer 2 (10 mM), 0.5 μl of Taq polymerase (Takara), 0.5 μl of RNasin (Promega), 0.5 μl of reverse transcriptase (Takara) and 35.2 μl of DEPC-treated H_2O were mixed. The reverse transcription reaction was performed at 42°C for 30 minutes and at 95°C for 5 minutes, and a cycle of 94°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute was repeated 40 times, followed by a reaction at 72°C for 7 minutes to obtain NK4 cDNA. NK4 cDNA thus obtained was cloned into pCRII™ vector using TA Cloning Kit (Invitrogen) to obtain pCRII/NK4. The primer used was the DNA fragment represented by SEQ ID NO:5 or 6.

(1-2) Construction of recombinant expression vector Ad-NK4

The replication-deficient adenovirus vectors used in this study are E1a-, partially E1b-, and partially E3-deleted vectors based on human adenovirus type 5. Briefly, an adenovirus vector was generated by homologous recombination of pJM17 plasmid (Microbix Biosystems Inc., Toronto, Canada) and shuttle

plasmid vector pCMV.SV2+ containing an expression cassette using the cytomegalovirus early promoter/enhancer, followed by human NK4 cDNA having a nucleotide sequence of SEQ ID NO:2 of the present invention and a polyadenylation signal. The resultant recombinant AdCMV.NK4 vector was purified by cesium chloride density gradient ultra-centrifugation. The total numbers of viral particles in the viral sample were measured by OD260 (where an OD260 of 1 is equal to 10^{12} particles). The titers (expressed as pfu per milliliter) of viral stocks were quantified by a plaque-forming assay using the human embryonic kidney 293 cells. AdNull,constructed previously, has no transgene and was used as a control.

(1-3)Establishment of epithelial cells of oral mucosa (OMEC)

Intraoral tissues were sampled from Wister rat with age 3 to 6 weeks and were subdivided. The tissue pieces were immersed twice in PBS (pH7.4.4, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing antibiotics (1000 U/ml of penicillin G potassium, 1 mg/ml of kanamycine and 2.5 μg/ml of amphotericin B). The tissue after immersion was immersed again in a DMEM culture medium (Gibco Laboratories Inc., Grand Island, NY) containing 0.2% dispase (Sigma-Aldrich Co., St. Louis, MO). Subsequently, the tissue was treated at room temperature for 30 minutes using a solution containing 0.25% of trypsin and 5mM EDTA, followed by washing with a DMEM culture medium containing 10% of FBS (CSL Ltd., Victoria, Australia). The sample tissue

obtained was stirred for 30 minutes in the DMEM culture medium containing 5% of FBS to release the cells, and the free OMEC cells were obtained by filtering with a filter with a pore size of 50 μm.

After treating Swiss 3T3 cells (Dainippon Pharmaceutical Co., Ltd.; Osaka, Japan) with 4 μg/ml of mitomycin C (Wako Pure Chemical Industries, Tokyo, Japan) for 2 hours, 1×10^5 cells were seeded on each well (Costar Inc., NY) of 6-well plate filled with EFM culture medium (DMEM culture medium: Ham's F culture medium (Nihonseiyaku, Tokyo, Japan)= 3:1) containing 10% CO_2. The 10% CO_2-containing EFM culture medium was supplemented with 5% FBS, 5 μg/ml insulin (Wako Pure Chemical Industries), 5 μg/ml transferrin (Wako Pure Chemical Industries), 2×10^{-9} M triiodotyrosine (Sigma-Aldrich Co.), 10 ng/ml cholera toxin (Sigma-Aldrich Co.), 0.5 μg/ml hydrocortisone (Wako Pure Chemical Industries), 100 U/ml penicillin, 0.1 mg/ml kanamycine and 0.25 mg/ml of amphotericin B.

Subsequently, 1×10^5 of free OMEC was seeded on each well. On day three after seeding the cell, 10 ng/ml of epidermal growth factor (human recombinant epidermal growth factor: Wako Pure Chemical Industries) was added to each well. After confirming that OMEC had grown to confluence after 7 to 10 days, the cells were subjected to passage culture. The second or third passage culture cells were harvested as established cells.

(1-4)Measurement of NK4 production by epithelial cells of oral

mucosa

OMEC cells of 2×10^5 cells were seeded in each well of a 12-well plate (Greiner Bio-one Co., Ltd.) coated with collagen type I. After adding 1ml of 2% FBS-containing DMEM culture medium, the cells were cultivated for 72 hours, followed by transfecting with Ad-NK4 at 10, 50, 100 and 200 MOI relative to 500μl of the culture medium. Thus OMEC into which Ad-NK4 is introduced was obtained. The supernatant of the culture medium was removed after the infection, and 1 ml of DMEM(Dulbecco's Modified Eagle's Medium) containing 2% FBS(fetal bovine serum) was added to each well. The culture supernatant was taken out at every 48 hours after the infection to measure the amount of secreted NK4 in the culture supernatant. OMEC not infected with Ad-NK4 was also cultured as a control, and the amount of secreted NK4 was measured. The concentration of NK4 was measured using IMMUNUS human HGF enzyme immunoassay kit (Institute of Immunology, Tokyo, Japan). Human recombinant NK4 was used as the standard in the ELISA.

(2)Production of NK4 by pancreatic cancer cells, lung carcinoma cells or melanoma cells

(2-1)Construction of recombinant expression vector

Ad-NK4 obtained as described in the columns (1-1) and (1-2) was used. Ad.LacZ expressing lacZ gene was similarly constructed with the proviso that LacZ gene was used instead of NK4 cDNA.

(2-2) procurement of cells

Human pancreatic cancer SUIT2 cells were generously provided by Dr. Iguchi (National Kyusyu Cancer Center, Fukuoka, Japan). Lewis lung carcinoma (LLC) cells and melanoma B16F10 cells were obtained from American Type Collection (Manassas, VA, USA).

(2-3) Measurement of NK4 production by pancreatic cancer cells, lung carcinoma cells or melanoma cells

Human pancreatic cancer SUIT2 cells, Melanoma B16F10 cells or Lewis lung carcinoma (LLC) cells of 2×10^5 cells were seeded in each well of a 12-well plate (Greiner Bio-one Co., Ltd.) coated with collagen type I. After adding 1ml of culture medium, the cells were cultivated for 72 hours, followed by transfecting with either Ad-NK4 or Ad-LacZ at 50 and 100 MOI relative to 500μl of the culture medium. Thus, SUIT2, B16F10 or LLC cells into which Ad-NK4 is introduced were obtained.

The supernatant of the culture medium was removed after the infection, and 1 ml of culture medium was added to each well. The culture supernatant was taken out at every 24 hours after the infection to measure the amount of secreted NK4 in the culture supernatant. The concentration of NK4 was determined by enzyme-linked immunosorbent assay (ELISA), using a kit ELISA kits for detection of human HGF (IMMUNUS, Institute for Immunology, B-Bridge International, Mountian View, CA, USA). Human recombinant NK4 was used as the standard in the ELISA.

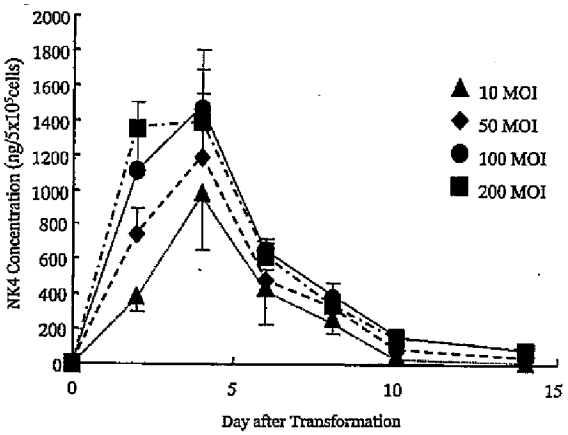
In the method described above, RPMI-1640 medium containing 10% FBS was used as culture medium for human pancreatic cancer SUIT2 cells and Lewis lung carcinoma(LLC) cells, and DMEM containing 10% FBS was used as culture medium for Melanoma B16F10 cells.

(B)Result

(1) NK4 production by epithelial cells of oral mucosa

The result is shown in Fig. 1 below. Values of Fig.1 represent the means ± SD.

Fig.1



When Ad-NK4 was infected to OMEC at 50 MOI, NK4 production in the medium was about 700ng/5×10^5 cells at 48 hours after infection. NK4 concentration of 700ng/5×10^5 cells is equivalent to 140ng/1×10^5 cells.

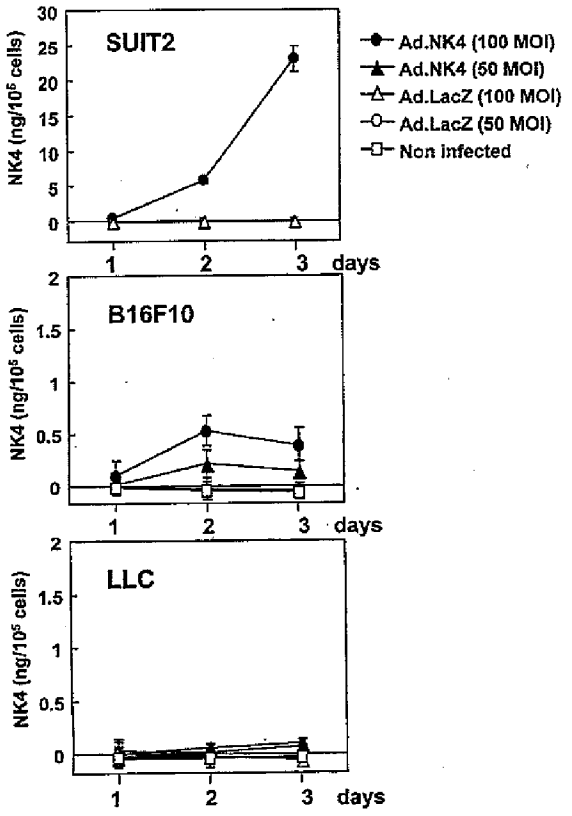
When Ad-NK4 was infected to OMEC at 100 MOI, NK4 production in the medium was about 1100ng/5×10^5 cells at 48 hours after

infection. NK4 concentration of 1100ng/5×10^5 cells is equivalent to 220ng/1×10^5 cells.

(2)-NK4 production by pancreatic cancer cells, lung carcinoma cells or melanoma cells

The result is shown in Fig.2 below. Values of Fig.2 represent the means ± SEM(n=3 in each group).

Fig.2



When Ad-NK4 was infected to Human pancreatic cancer SUIT2 cells at 50 MOI, NK4 production in the medium was scarcely detected. When Ad-NK4 was infected to Human pancreatic cancer

SUIT2 cells at 100 MOI, NK4 production in the medium was about 5ng/1x10^5 cells at 48 hours after infection.

When Ad-NK4 was infected to Melanoma B16F10 cells at 50 MOI, NK4 production in the medium was about 0.2ng/1x10^5 cells at 48 hours after infection. When Ad-NK4 was infected to Melanoma B16F10 cells at 100 MOI, NK4 production in the medium was about 0.5ng/1x10^5 cells at 48 hours after infection.

When Ad-NK4 was infected to Lewis lung carcinoma(LLC) cells at 50 MOI or 100 MOI, NK4 production in the medium was slightly detected at 48 hours after infection.

(C) Discussion

NK4 production(ng/1x10^5 cells) by each of the above 4 types of cells which were infected with Ad-NK4 is summarized as follows.

(Unit: ng/1x10^5 cells)

	Epithelial cells of oral mucosa	Human pancreatic cancer SUIT2 cells	Melanoma B16F10 cells	Lewis lung carcinoma(LLC) cells
50 MOI	140	Not Detected	0.2	slight
100 MOI	220	5	0.5	slight

As a result, it is evident that production of NK4 by epithelial cells of oral mucosa into which DNA encoding NK4 has

been introduced is 44-fold or more greater than that by pancreatic cancer cells, lung carcinoma cells or melanoma cells.

It is declared by the undersigned that all statements made herein of undersigned's own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18U.S.C.1001, and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

Date: Oct 7, 2009

Kunio MATSUMOTO